ASSESSING DENDRITIC CELL ACTIVATION AND PHENOTYPIC RESPONSES TO GOLD NANOPARTICLE TREATMENTS IN VITRO

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ASSESSING DENDRITIC CELL ACTIVATION AND PHENOTYPIC RESPONSES TO GOLD NANOPARTICLE TREATMENTS IN VITRO

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TABLE OF CONTENT

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ab: antibody

APC: antigen presenting cell

AuNP: gold nanoparticle

CD86: cluster of differentiation 86

DC: dendritic cells

DC-SIGN: dendritic cell-specific intercellular adhesion molecule-3-grabbing non integrin

GM-CSF: granulocyte-macrophage colony-stimulating factor

iDC: immature dendritic cell

IMF: inflammatory maturation factor

IL: interleukin

ILT3: immunoglobulin-like transcript 3

LPS: lipopolysaccharide

mDC: mature dendritic cell

MHC: major histocompatibility complex

NP: nanoparticle

PBMC: peripheral blood mononuclear cells

PBS: phosphate buffered saline

PEG: polyethylene glycol

tDC: tolerogenic DCs

TMF: tolerogenic maturation factor

TNF: tumor necrosis factor

Tregs: regulatory T-cells

SUMMARY

Dendritic cells (DCs) are potent antigen-presenting cells (APCs) that orchestrate an immune response to variety of pathogens or initiate tolerance. Successful manipulation of DCs to control the immune system is a powerful approach for optimizing vaccine delivery, tissue engineering, or immunotherapeutic approaches. Biomaterials are widely used in combination products for tissue regeneration or vaccine delivery; however knowledge of how biomaterials harness DC surface receptors to induce phenotypic changes is still lacking, particularly for nanoparticles (NPs). In this study, a point of reference is established for the immunogenic effects that gold nanoparticle (AuNP) parameters have on DCs. The overall goal of this research is to use this information to engineer a specific immune response. Preliminary results indicate a concentration dependent relationship between increasing levels of AuNPs and pro-inflammatory/maturation of DCs for the bare, and Polyethylene glycol (PEG) coated nanoparticle treatment groups. AuNPs that were serum coated had the opposite trend, where an increasing concentration of AuNPs produced a tolerogenic DC phenotype. Additionally, the serum-coated AuNP treatment group showed the highest levels of tolerogenic DC expression for all treatment groups, across all concentrations. There appears to be no significant difference in the levels of induced mature DC activation between Bare, PEG-2K (short chain PEG), and PEG-5K (long chain PEG).

CHAPTER 1: INTRODUCTION

NPs have become increasingly investigated in application to vaccine and immunotherapy research. There are several benefits to a NP-based approach, including targeted delivery of antigen, controlled drug release, improved transport kinetics, tracking capability via medical imaging, and multitherapeutric loading [1]. Additionally, NPs can be engineered to act as adjuvants themselves, specifically inhibiting or enhancing immune responses depending on their variable physical properties.

One of the most promising applications of NPs in immunotherapy pertains to NP interaction with dendritic cells (DCs). However, the potential of NPs to modulate a specific immune response through inducing an activated or tolerogenic DC phenotype has only been recently explored, and is not well understood.

The objective of this thesis research was to determine relationships between DC phenotype and NP physical properties. The primary hypothesis was that different combinations of gold nanoparticle (AuNP) parameters trigger differential DC responses, leading to distinct DC activation profiles. Through collaboration with Dr. Warren Chan and PhD candidate James Lazarovits at the University of Toronto, AuNP samples of distinct size, coating, and concentration were used to study DC phenotype. DC response was assessed by maturation marker expression and tolerogenic factor expression using fluorescent spectroscopy.

CHAPTER 2: RESEARCH SIGNIFICANCE

Cancer and autoimmune diseases are among the most prevalent diseases in the United States and the world. In the United States, over 1.6 million new cases of cancer were estimated for 2016, resulting in 500,000 deaths [2]. This year, over 600,000 Americans are expected to die of cancer, more than 1,650 people a day [3]. Furthermore, national expenditure for cancer care totaled nearly $125 billion in 2010, and is projected to reach as high as $156 billion in 2020 [2].

Autoimmune diseases are even more prevalent in the U.S., affecting more than 50 million Americans, and are the second highest cause of chronic illness in the nation [4]. Most autoimmune diseases are debilitating, require a lifetime of treatment and can be life-threatening. Because of the high number and variety of prognoses associated with autoimmune diseases, a clear national expenditure has been difficult to determine; however, in 2001 the National Institutes of Allergy and Infectious Diseases reported annual autoimmune disease treatment costs to be greater than $100 billion, and projected to increase [4].

High morbidity and diagnosis rates for cancer and autoimmune diseases, in addition to increasing health care costs are driving the industry to develop cures and more effective treatment. One of the greatest hurdles to developing cancer immunotherapies is the widespread induced immune tolerance at the tumor site. Antitumor responses elicited by the body or current immunotherapy treatments are subdued by the low immunogenicity of tumor antigens, proliferation of immunosuppressive cells (e.g. regulatory T-cells), and increased production of immunosuppressive cyotkines (IL-10, TGF-beta) [5]. An effective antitumor immune response requires the sustained activation

and proliferation of tumor-specific effector T-cells. Antigen presenting cells (APCs), specifically dendritic cells (DCs), uniquely influence T-cell activity to bridge innate and adaptive immunity, making them key cells in generating tumor-specific immunity [6]. Therefore, by manipulating DC activity and enhancing the capture of tumor-antigens by DCs, a tumor-specific immune response can be actualized. Engineering NPs to target DCs, deliver specific antigens, and function as adjuvants themselves could overcome the current challenges of cancer immunotherapeutic techniques.

The defining problem of autoimmune diseases is that the immune system responds to self-molecules as if they are foreign entities, attacking the body's own healthy cells and tissues. Immunosuppressant therapies to control this flawed immune response are critical for treating autoimmune diseases, such as multiple sclerosis, lupus erythematosus, or rheumatoid arthritis [7]. However, immunosuppressant drugs currently used are nonspecific, interfere with larger pathways and cells, have toxic effects, and increase patient susceptibility to infectious diseases [8, 9]. NPs can potentially ameliorate these severe side effects by delivering the optimal amount of immunotherapy to a specific site with controlled dosing [7], and through their ability to mediate an immune response by manipulating DC activation.

NPs have already shown promising immunomodulatory effects due to their unique physical properties (size, shape, charge, coating etc) which can be engineered to optimize biodistribution, site-specific targeting, immunogenicity, and therapeutic loading [31]. However, the relationship between different combinations of NP parameters and their impact on the immune system is poorly understood.

The objective of this research was to gain understanding of how DCs, which are central to directing immune responses, integrate signals from multiple stimuli. This research is expected to make important contributions to the fields of immunology and biomaterials science. Through this research we will gain important insights in rationally design the DC environment, particularly using engineered NPs, to direct the DC phenotype to influence immune responses in clinical situations such as transplantation, cancer and autoimmune disease.

CHAPTER 3: LITERATURE REVIEW

3.1 Innate and Adaptive Immunity

The immune system is categorized into two parts, the innate and adaptive responses. While there is much interaction between the two responses, they are differentiated based on the speed and specificity of the reaction. The innate immune response includes physical, chemical, and microbiological barriers, as well as other elements that provide an immediate, but non-specific host defense. Adaptive immunity is more precise, but can take several days or weeks to develop. This adaptive response has antigen-specific reactions through T lymphocytes and B lymphocytes, and has memory so that subsequent exposure has a more rapid and vigorous response [10]. To understand how the innate and adaptive immune responses are initiated and linked, it is important to understand the biology and function of dendritic cells.

3.2 Dendritic Cells

Dendritic Cells (DCs) are antigen presenting cells (APCs) that are activated to mediate a host immune response. They are integral in both adaptive and innate immunity through their role in T cell activation. The principal functions of DCs are to capture and present antigens to other cells in the immune system. To accomplish this, DCs phagocytize antigens, process them internally, and present the resulting antigen peptide on their cell surface. They are found in nearly all tissues and organs, including lymphoid organs such as the thymus, spleen and lymph nodes. The activity of DCs in stimulating specific T cell responses has shown potential in developing new vaccine strategies for the

treatment of a variety of ailments, including infections, allergic and autoimmune diseases, and cancer [11].

DCs originate from a myeloid progenitor cell in the bone marrow, which can differentiate into many cell types, including monocytes. Millions of monocytes are produced daily, and normally circulate in the bloodstream for a short time before undergoing spontaneous apoptosis [12]. However, during an innate immune response, monocytes are recruited to the site of inflammation by chemoattractant proteins that are secreted by leukocytes at the site [13]. There they infiltrate into the tissue and differentiate into immature dendritic cells (iDCs) when in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) [14].

Initially, chemoattractants resulting from tissue damage, pathogens products, and inflammatory cytokines attract iDCs to the site of infection. The iDCs phagocytize antigens, degrade them endocytically, and produce antigenic peptides capable of binding to major histocompatibility complex class I (MHC I) and MHC II molecules. When these surface molecules are synthesized and expressed on the DC surface, the phenotype of the cell changes from an iDC to a mature dendritic cell (mDC). The function of the DC also changes from antigen capturing cells to antigen presenting cells (APCs). mDCs lose responsiveness to inflammatory chemokines, and gain responsiveness to lymphoid chemokines, which direct their migration to the draining lymph node [15]. In the lymph nodes, DCs physically interact with T-cells to activate, or suppress, an adaptive immune response.

3.3 T-cell Differentiation

For T-cell activation to occur, an immature, or naive T-cell must recognize a foreign peptide bound to a MHC molecule while simultaneously receiving a co-stimulatory signal from an APC. DCs are one of the few APCs capable of expressing both MHC I and MHC II molecules, as well as the co-stimulatory cell-surface molecule necessary for the differentiation of T-cells into effector cells and their proliferation. DCs with MHC I molecules on their surface stimulate CD8+ T-cells (cytotoxic T-cells), and DCs with MHC II molecules stimulate CD4+ T cells (helper T-cells) [15], thus initiating an adaptive immune response.

DCs can also suppress activation of the immune system by inducing the differentiation of native T-cells to regulatory T-cells. These tolerogenic DCs (tDCs) have low levels of costimulatory molecules, so they provide insufficient stimulatory signals for the naive T-cells to differentiate into effector cells. The naïve T-cells instead differentiate into regulatory T-cells (Tregs), which downregulate the induction and proliferation of effector T-cells, thereby inducing an immunosuppressive response [16].

Because of the integral role DCs play in the activation of T-cells, inducing a particular DC phenotype can induce a corresponding immunoactiving or immunosuppressive response. Therefore, finding an effective means of controlling DC phenotype is crucial to advancing immunoengineering and immunotherapy based treatments of diseases, ranging from autoimmune diseases to cancer.

3.4 Nanoparticles

Nanoparticles are defined as objects that range in size from 1-100nm. The specifications of nanoparticles vary widely, with different sizes, shapes, materials, and surface charges possible. Some unique advantages of using nanoparticles in medicine include the ability of nanoparticles to function as carriers, target specific tissues or cells, control the release of drugs, and exhibit low cell toxicity [17].

3.5 Gold Nanoparticles (AuNPs)

While Gold (Au) compounds have been used as therapeutic agents for the treatment of immune-based diseases for decades, gold nanoparticles (AuNPs) have emerged as promising new drugs for immunotherapy treatment. AuNPs are well suited for biological applications because of their inertness, water solubility, and nontoxicity. They have been used to carry antigens for vaccines and stimulate antigen presentation, are actively ingested by phagocytes, and can function as adjuvants [17]. AuNPs have been used in gene therapy as well, regulating gene transcript by delivering oligonucleotides or small RNA molecules into cells [18]. AuNPs have even been used to enhance x-ray imaging and radiotherapy treatment of cancer [19].

Despite these medical advances however, the optimization and adverse effects of AuNPs are not well understood, and different studies often yield contradictory results. The effects of several variables involving the physiochemical characteristics of nanoparticles are poorly described, including size, shape, surface area, coating and concentration [20]. Elucidating these properties and their cellular interactions is critical for the safe and effective application of AuNPs in nanomedicine.

3.6 Application of AuNPs to Immunotherapy

Understanding the interaction of NPs with the immune system is critical to optimize and develop therapeutic AuNP treatments. A great deal of research has been done to modulate immune responses using DCs, specifically because of their antigen presenting capabilities. AuNPs have been studied as potential carriers in application of antigen delivery to DCs. Antigens related to viruses and tumors in particular have been studied, with results indicating the successful targeted delivery of antigen to DCs through AuNP carriers [21, 22]. However, AuNPs do not require the presence of an antigen to have immunostimulatory effects on DCs and macrophages [23].

3.6.1 Effect of Nanoparticle Size

The size of the NP can influence the efficiency of cellular uptake. NPs that mimic pathogens in size and appearance enhance uptake by DCs, which have evolved to recognize pathogens of a particular size, such as viruses and bacteria [24]. Studies have shown an inverse relationship between the size of the particle and the efficiency of particle uptake by DCs [25, 26]. However, it can be difficult to determine an ideal particle size because the NPs must be large enough to load if to be used as carriers, but particles that are too large are more likely to be eliminated before interacting with DCs.

Data regarding the impact of NP size on immunological functions has been contradictory depending on the specifications of the NPs used in a given experiment. Small AuNPs (3 nm in diameter) have been shown to be noncytotoxic and nonimmunogenic with macrophages, in that the AuNPs did not elicit the secretion of proinflammatory cytokines TNA-alpha and IL1-beta [27]. Non-cytotoxicity has also been

shown for DCs and macrophages with lager AuNPs (10 nm and 60 nm in diameter) even at high concentrations [28, 29]. However, a contradictory study showed that smaller AuNP sizes were associated with increased expression of proinflammatory genes IL-1, IL-6 and TNA-alpha, as well as higher cytotoxicity when compared to silver NPs [30]. This size-dependent toxicity where smaller AuNPs have greater cytotoxicity has been observed in various cell lines [31]. Conflicting results such as these demonstrate the need for more optimization studies to clearly understand how particle size affects cellular interactions with the NP.

3.6.2 Effect of Nanoparticle Coating

One method by which NPs can affect an immune response is through the phagocytosis of NPs by APCs, including macrophages and DCs. NP coatings can be applied to enhance or avoid uptake by APCs, and both results have important implications for how effectively a desired immune response can be induced. For example, attaching long chain polymers, such as polyethylene glycol (PEG), has been shown to decrease phagocytic uptake of NPs, thus prolonging their circulation time [32]. A similar decrease in cellular uptake has been shown with serum-coated AuNPs [33]. This result is important when targeting NPs to specific tissues or cells, such as for certain cancer treatments, where the sequestering of nanoparticles via phagocytosis can cause insufficient dosage to the target site. In this case, the ability of NPs to avoid the immune system increases their target efficiency.

On the other hand, enhanced phagocytosis of NPs is ideal for situations where APCs are targeted and utilized to induce a general immune-activating or immunosuppressive

response. In this case, NP coatings could be used to enhance phagocytosis, such as the facilitation of targeted antigen delivery to DCs by conjugating ligands to the surface of NPs for pattern recognition receptors [34]. NPs can also be designed to induce specific DC phenotypes (iDC, mDC, or tDC) upon DC phagocytosis of the particles. Through this mechanism, NPs can be used to engineer a DC-mediated, specific immune response by controlling the differentiation of T-cells into effector or regulatory cells.

Not only can different coatings give NPs different chemical properties, but different densities of the same coating can also cause a change in the affect NPs have. For example, a study with using AuNPs and increasing PEG coating densities showed that macrophage uptake efficiency was high and serum-dependent at low PEG densities, and less efficient and serum-independent at high PEG densities [34].

Another important implication of selecting a NP coating pertains to the formation of an AuNP-protein corona. This dynamic biopolymer layer is important because it forms the first nano-bio interface, thus determining how the AuNPs interact with living cells [35]. As many as 69 plasma protein can bind to the AuNP surface [36, 37], and these proteins largely determine the AuNP's fate in the body through biodistribution, efficiency of cellular uptake and clearance, and immunological properties.

3.6.3 Effect of Nanoparticle Concentration

The concentration of AuNPs can play a significant role in immunological interactions, including cellular uptake, immune activation, and cytotoxicity, but most research regarding NP concentration specifically has focused on the potential toxicity of AuNPs. Increasing AuNp concentrations have been shown to result in increased rates of

acute toxicity and apoptosis, independent of changes in particle size [38, 39]. However, the threshold at which concentrations of NPs with particular specifications (size, coating etc) result in cytotoxicity is unclear.

CHAPTER 4: DC RESPONSES TO GOLD NANOPARTICLE TREATMENTS

4.1 Materials and Methods

Methods adapted and modified from Kou PM and Babensee JE. Validation of a high-throughput methodology to assess the effects of biomaterials on dendritic cell phenotype. Acta Biomaterialia. 6:2621-2630 (2010) [40].

4.1.1 Derivation of immature DCs (iDCs)

Human peripheral blood was collected from consenting volunteers at the Student Health Phlebotomy Laboratory in accordance with protocol H10011 or H15072 approved by the Institutional Review Board of the Georgia Institute of Technology. The whole blood was heparinized (333 U/mL blood) (Abraxis Pharmaceutical Products, Schaumburg, IL) and diluted in a 1:1 ratio with phosphate buffered saline (PBS). Peripheral blood mononuclear cells (PBMCs) were then isolated by centrifugation using lymphocyte separation medium (Cellgro MediaTech, Herndon, VA). Erythrocytes were lysed using red blood cell lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA), and after several washing steps using PBS, the PBMCs were resuspended at a concentration of $5x10^6$ cells/ml in DC media, comprised of filter-sterilized RMPI-1640 containing a final concentration of 10% (v/v) heat inactivated fetal bovine serum (Cellgro MediaTech, Herndon, VA) and 100 U/ml of Penicillin/Streptomycin (Cellgro MediaTech). The cells were then plated in a volume of 10 ml/plate in a tissue-culture dish (Primaria 100 x 20 mm2 tissue-culture dish, Becton Dickinson, Franklin Lakes, NJ) and incubated for 2 hours at 95% relative humidity and 5% CO_2 at 37◦C. Following

incubation, the dishes were washed three times with warm DC media to remove non-adherent cells. New warm media was added to the remaining adherent cells at a volume of 10ml/plate, and supplemented with 1000 U/ml GM-CSF and 800 U/ml IL-4 (PeproTech, Rocky Hill, NJ). The cells were then incubated for a period of 5 days at 95% relative humidity and 5% CO_2 at 37◦C without changing the media to induce the differentiation of monocytes into iDCs.

4.1.2 Human serum extraction

On day 0 of DC culture, human peripheral blood was collected from consenting volunteers at the Student Health Phlebotomy Laboratory in accordance with protocol H10011 of H15072 approved by the Institutional Review Board of the Georgia Institute of Technology. Blood collected for serum extraction was from the same donor as in section 4.2.1. The blood was allowed to clot for 30 minutes at 25◦C, then centrifuged for 10 minutes at 2000g. The resulting supernatant contained the serum, which was extracted and stored at 0◦C for future use on day 5 of DC culture.

4.1.3 AuNP treatments preparation

Gold nanoparticles (60 nm) were kindly supplied by the University of Toronto (Toronto, Canada) as stock solutions containing uncoated (bare), PEG-2K coated and PEG-5K coated AuNPs. All stock solutions had an AuNP concentration of 5 nM. The bare AuNPs were suspended in ultrapure water and PEGylated AuNPs were suspended in PBS in their respective stock solutions.

Bare, PEG-2K and PEG-5K coated AuNP treatment preparation

32 µL of each stock solution were aliquoted; two aliquots of the bare AuNP stock were prepared, one for the final bare AuNP treatment and one for the final serum-coated AuNP treatment. The four aliquots were centrifuged for 35 minutes at 1200g to pellet the AuNPs, then each aliquot was washed twice with sodium citrate tribasic dehydrate solution (200 µL, 5 mM), centrifuging samples for 35 minutes at 1200g and aspirating the supernatant after each wash. The resulting AuNP pellets of one of the bare AuNP aliquots, the PEG-2K AuNP aliquot and the PEG-5K AuNP aliquot were resuspended in 1 mL RPMI media, for an AuNP treatment sample concentration of 160 pM. A serial dilution was performed on these three of the aliquots to obtain three concentrations (160 pM, 16 pM, and 1.6 pM) for each AuNP coating type.

Serum-coated AuNP treatment preparation

The pellet in the remaining aliquot of bare AuNPs was resuspended in 40 µL of chilled PBS, and added to an Eppendorf tube containing 400 µL of human serum (filtered via 0.22 µm PES filter). The AuNPs were incubated in human serum for 1 hour with 95% relative humidity and 5% CO_2 at 37◦C. After incubation, the serum-AuNP solution was centrifuged for 35 min at 1200g and the supernatant was aspirated. The serum-coated AuNPs were washed with chilled PBST twice by adding 750 ul chilled PBST, centrifuging for 35 min at 1200g, and aspirating the supernatant. After the second PBST wash, the serum-coated AuNPs were washed with 1 mL chilled PBS by the same process. The final serum-coated AuNP pellet was resuspended in 1 mL RPMI media, for an AuNP

treatment sample concentration of 160 pM. A serial dilution was performed to obtain three concentrations (160 pM, 16 pM, and 1.6 pM) of serum-coated AuNP solutions.

4.1.4 Treatment of iDCs with AuNPs in 96-well plates

On day 5 of DC culture, NP treatments were prepared as described in section 4.2.3. Loosely adherent and non-adherent iDCs were harvested and resuspended in DC media with 1000 U/ml GM-CSF and 800 U/ml IL-4 at $5x10^5$ DCs/ml. 100 µL of iDCs at a concentration of 5×10^5 DCs/ml were plated onto each well in the 96-well tissue culture plate (Corning). The wells for the negative control of iDCs remained untreated, the wells for the positive control of mDCs were treated with LPS (1 mg/mL; *E. coli* 055:B5; Sigma), and the wells for the positive control of tDCs were treated with human IL-10 (3,500 U/mL; R&D Systems) and human IFN-alpha (35,000 U/mL; R&D Systems). 150 µl of each concentration group for each AuNP coating treatment group were added to different wells of the 96-well plate containing iDCs, such that the final concentration of AuNPs in each well were 0.1 pM, 1.0 pM and 10 pM (Figure 1). The iDCs were then incubated with the control, bare AuNP, PEG-2K coated AuNP, PEG-5K coated AuNP, and serum coated AuNP treatments for 24 hours with 95% relative humidity and 5% CO_2 at 37◦C.

On day 6, the DCs treated in the 96-well plate were transferred directly to wells of a 96-well black filter plate wetted with PBS. The supernatants were removed by centrifuging the filter plate for 4 minutes at 400rpm. To each well, 100 µl of cold working fixation solution (0.05 % paraformaldehyde) was added, and the plate was incubated for at least 30 min at room temperature on a microplate shaker at 600 rpm

(VWR, West Chester, PA) followed by the removal of the fixative by centrifugation (4 minutes, 400 rpm). Subsequently, DCs were stained with antibodies for surface marker expression, namely, anti-DC-SIGN-FITC (Clone 120507; R & D Systems), anti-CD86-PE (Clone BU63; Ancell) and anti-ILT3-AF647 (Clone ZM4.1, Biolegend). For isotype staining for background fluorescence elimination, the following antibodies were used: IgG2B-FITC (clone 133303; R&D Systems), IgG1-PE (clone MOPC31C; Ancell) and IgG1κ (clone MOPC-21; Biolegend) (Figure 1).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Antibody 150 ul/well iDC			Isotyp 150 ul/well iDC			Antibody 60nm - 150 ul 0.1pM Au(PEG5K)			Cells-Back-ground	Cells-Back-ground	Cells-Back-ground
B	Antibody 150 ul/well mDC			Isotyp 150 ul mDC			Isotyp 60nm - 150 ul 0.1pM Au(PEG5K)			Media	Media	Media
C	Antibody 150 ul/well tDC			Isotyp 150 ul tDC			Antibody 60nm - 150 ul 1pM Au(PEG5K)			Isotyp 60nm - 150 ul 1pM Au(PEG5K)		
D	Antibody 60nm - 150 ul 0.1pM Au			Isotyp 60nm - 150 ul 0.1pM Au			Antibody 60nm - 150 ul 10pM Au(PEG5K)			Isotyp 60nm - 150 ul 10pM Au(PEG5K)		
E	Antibody 60nm - 150 ul 1pM Au			Isotyp 60nm - 150 ul 1pM Au			Antibody 60nm - 150 ul 0.1pM Au(PEG2K)			Isotyp 60nm - 150 ul 0.1pM Au(PEG2K)		
F	Antibody 60nm - 150 ul 10pM Au			Isotyp 60nm - 150 ul 10pM Au			Antibody 60nm - 150 ul 1pM Au(PEG2K)			Isotyp 60nm - 150 ul 1pM Au(PEG2K)		
G	Antibody 60nm - 150 ul 0.1pM Au(Serum)			Isotyp 60nm - 150 ul 0.1pM Au(Serum)			Antibody 60nm - 150 ul 10pM Au(PEG2K)			Isotyp 60nm - 150 ul 10pM Au(PEG2K)		
H	Antibody 60nm - 150 ul 1pM Au(Serum)			Isotyp 60nm - 150 ul 1pM Au(Serum)			Antibody 60nm - 150 ul 10pM Au(Serum)			Isotyp 60nm - 150 ul 10pM Au(Serum)		

Figure 1. Layout of AuNP-DC treatments in 96-well plate. AuNP concentrations of 0.1 pM, 1.0 pM and 10 pM were added to DCs in individual wells for all AuNP treatment groups (bare, serum coated, PEG-2K coated, and PEG-5K coated AuNPs). Antibody staining was added to three of the six wells used for each treatment, and isotype staining was added to the other three wells.

The plate was incubated in a plate shaker (600 rpm) at 40ºC for 40 minutes protected from light. After this staining procedure, DCs were washed three times with washing solution of 0.1% BSA and 2mM EDTA in PBS, pH 7.20 by centrifugation at 400 RCF for 4 min. Afterwards, 100 µl of the washing solution was added to DCs and the fluorescence of each treatment group were measured. The geometric mean fluorescent intensities were measured with a Tecan Infinite F500 microplate reader (Tecan US, Durham, NC) using excitation filters of 535/25 and 485/20 and the emission filters of 590/20 and 535/25, for PE and FITC, respectively, and 650/668 for anti-ILT3 - AF647.

The surface marker, CD86, is a costimulatory molecule for which expression becomes up-regulated upon DCs maturation. Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) is an endocytic receptor which expression is not significantly down-regulated upon DCs maturation. Immunoglobulin-like transcript 3 (ILT3) is an inhibitory receptors which expression is up-regulated upon anti-inflammatory (tolerogenic) DC response. The ratio of respective geometric mean fluorescent intensities for CD86 expression divided by DC-SIGN expression defines the metric of "inflammatory maturation factor" (IMF) as an indicator of pro-inflammatory DC phenotype. The ratio of respective geometric mean fluorescent intensities for ILT3 expression divided by CD86 expression defines the metric of "tolerogenic maturation factor" (TMF) as an indicator of tDC phenotype.

4.2. Results

4.2.1 DCs treated with serum coated AuNPs had the greatest tolerogenic maturation factor expression

The TMF, as defined by the ratio of the geometric mean fluorescent intensities for ILT3/CD86, was determined for each AuNP coating type and concentration. As expected, the DCs treated with IL-10 and IFN-alpha to act as the positive tDC control had a significantly higher TMF than both the iDC and mDC controls, and the mDC control had a very low TMF (Figure 2). The TMF for the tDC control was higher than DCs treated with any concentration of bare, PEG-2K, or PEG-5K coated AuNPs. However, all concentrations of the serum-coated AuNP treatments showed a higher TMF than any other treatment group, including the tDC control. The PEG-5K coated AuNP treatment group of DCs had TMFs that were lower than the iDC control for AuNP concentrations 0.1 pM and 10 pM; at 1 pM, the TMF was higher than that of the iDC control. The bare AuNP treatment, serum coated AuNP treatment, and PEG-2K treatment showed a slight concentration dependence with increasing concentrations of AuNPs and an increased TMF (Figure 2).

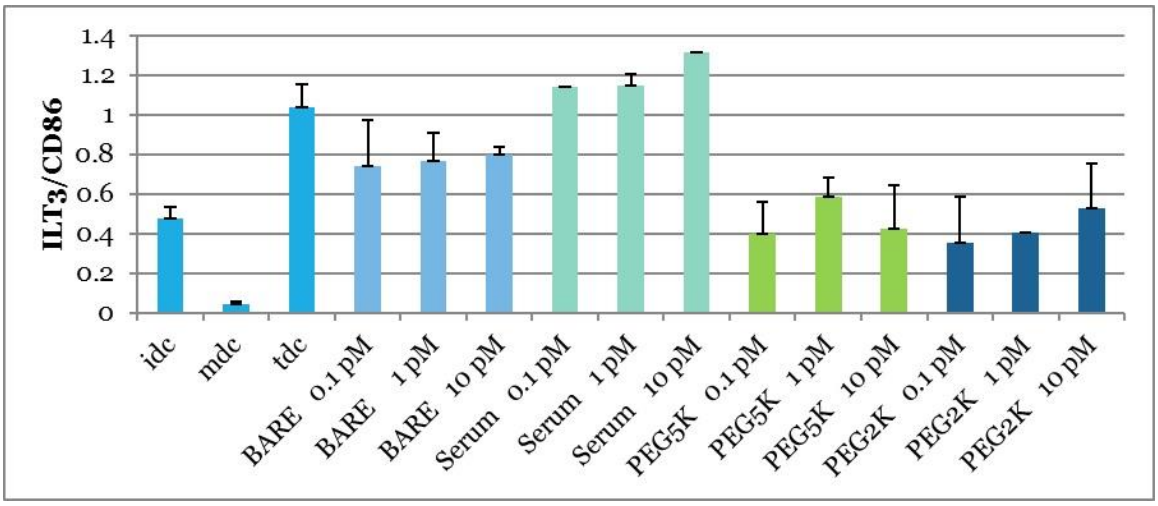

Figure 2. Tolerogenic maturation factor (ILT3/CD86) of DCs treated with AuNPs with mean ± range, n=2 donors. Treatments of different AuNP coatings (bare, serum, PEG-2K, PEG-5K) and concentrations (0.1 pM, 1.0 pM, 10 pM) were used. DCs were incubated with AuNP treatments for 24 hours at 37°C. DCs were stained with antibodies for surface marker expression, stained with isotype for background and geometric mean fluorescent intensities were determined for ILT3 and CD86 expression for each treatment group using a Tecan Infinite F500 microplate reader.

4.2.2 Inflammatory maturation factor expression showed concentration dependence for DCs treated with bare AuNPs and serum-coated AuNPs

The IMF, as defined by the ratio of the geometric mean fluorescent intensities for CD86/DCSIGN, was determined for each AuNP coating type and concentration. As expected, the positive control for inflammatory DC maturation (mDC) had the highest IMF, and the positive control for tolerogenic DC maturation (tDC) had the lowest IMF. DCs treated with the bare AuNP treatment group showed a strong positive correlation with increasing bare AuNP concentration and an increased IMF. The reverse trend was seen with the serum coated AuNP treatment group, where increasing concentrations of serum coated AuNPs were correlated to a decrease in the IMF (Figure 3). This apparent

concentration dependence for the serum coated AuNPs was seen with the TMF as well, with increasing concentrations of AuNPs correlating to an increased TMF (Figure 2).

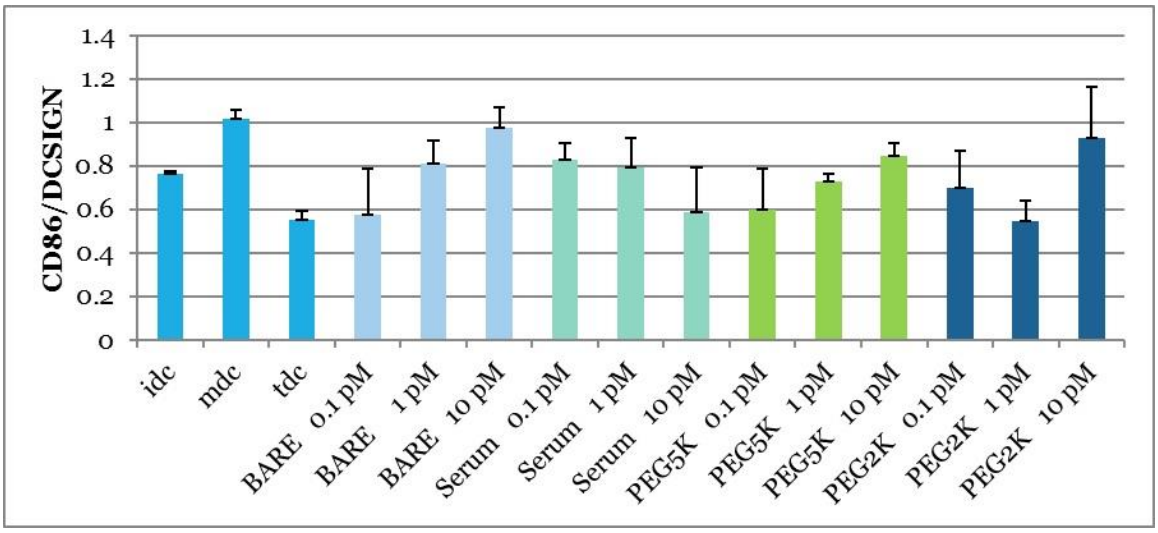

Figure 3. Inflammatory maturation factor (CD86/DCSIGN) of DCs treated with AuNPs with mean ± range, n=2 donors. Treatments of different AuNP coatings (bare, serum, PEG-2K, PEG-5K) and concentrations (0.1 pM, 1.0 pM, 10 pM) were used. DCs were incubated with AuNP treatments for 24 hours at 37°C. DCs were stained with antibodies for surface marker expression, stained with isotype for background and geometric mean fluorescent intensities were determined for CD86 and DCSIGN expression for each treatment group using a Tecan Infinite F500 microplate reader.

4.3 Discussion

The phenotype of DCs was differentially modulated by bare, serum-coated, PEG-2K coated, and PEG-5K coated AuNPs. Specifically, serum coated AuNPs had the highest levels of TMF (ILT3/CD86) for all concentration groups (0.1 pM, 1.0 pM, 10 pM), and all concentrations of serum coated AuNPs were also higher than the TMF for the tDC control. Additionally, the serum coated AuNPs showed concentration dependence for both the TMF and IMF values; increasing the concentrations of serum coated AuNPs increased the TMF and decreased the IMF. This indicates that the interaction between serum coated AuNPs and DCs resulted in greater levels of ILT3 expression on the DCs and a tolerogenic phenotype. The bare AuNPs also showed a

concentration dependent effect but for IMF expression, where increasing bare AuNP concentrations showed an increased IMF. However, the IMFs for all bare AuNP concentrations were still lower than the positive control, mDC. These results indicate that in the presence of bare AuNPs, DC expression of CD86 increases, therefore causing an inflammatory, activated DC phenotype.

The PEG-5K treatment group did not show concentration dependence for TMF, and the TMF values of all concentrations were closest to the iDC control. While concentration dependence was seen for PEG-5K coated AuNPs for IMF levels, these values were also closer to the iDC control than tDCs or mDCs. The PEG-2K coated AuNP treatment stayed within a similar range of TMFs and IMFs as the PEG-5K coated AuNP group. However, a slight concentration dependence was shown for the PEG-2K AuNP treatment for TMF, but not for IMF. These results indicate that the PEG-2K and PEG-5K AuNP coatings did not show a significant change in DC phenotype towards an activating or tolerogenic phenotype.

It is important to note that this data was accumulated from only two donors. More trials are necessary to demonstrate the statistical significance of the trends observed in these preliminary results for bare and serum coated AuNPs, as well as to demonstrate that the PEGylated AuNPs did not have a significant effect on DC phenotype. Additionally, an assumption being made in the analysis of the data in this study is that the AuNPs are actually being phagocytized by the DCs, which may not necessarily be the case. Because this is an *in vitro* study, DCs may simply be interacting with serum molecules and NPs in solution because there is not a complicated matrix of additional molecules and cells that would be found *in vivo*. In subsequent studies, the uptake of NPs by DCs should be

measured using Inductively Coupled Plasma-Atomic Emissions Spectroscopy (ICP-AES) for gold and magnesium content. DC responses to NPs will also be further characterized by cytokine profile analysis using Mulliplex bead technology.

5 Conclusion and Future Work

The data in this study indicates a strong tolerogenic inducing effect for DCs treated with serum-coated AuNPs, concentration dependence for an increased tolerogenic phenotype with increasing concentrations of serum coated AuNPs, and concentration dependence for an increased inflammatory phenotype with increasing concentrations of bare AuNPs. Additional experimental runs to increase donor number are needed to show statistical significance for the preliminary results shown here.

Future work should include examination of NP treatment variables such as using a greater range of concentrations to explore the extent of apparent concentration dependence (0.1pM – 100pM), using different sizes of AuNPs to assess the impact that NP size has on DC phenotypic changes (15nm, 60nm, 100nm), and testing a range of DC-AuNP exposure times (1 hour, 12 hours, 24 hours). Additionally, subsequent studies should be performed to further study the effect that AuNPs have on DCs and mechanism by which AuNPs alter DC phenotype, including cytotoxicity analysis using Vybrant Cytotoxicity Assay (Molecular Prodes, Eugene, OR), cytokine profile analysis using Mulliplex bead technology, and DC imaging to assess the extent of AuNP internalization using Inductively Coupled Plasma-Atomic Emissions Spectroscopy (ICP-AES) for gold and magnesium content.

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